EPR Spectroscopic Reinvestigation of the Activation of Iron Complexes of PMAH as a Bleomycin Model

István Lippai, Richard S. Magliozzo, and Jack Peisach*

Contribution from the Department of Physiology and Biophysics, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

Received February 10, 1998. Revised Manuscript Received October 9, 1998

Abstract: The structure and reactivity of iron complexes of PMAH, which contains ligands that mimic the metal binding domain of iron bleomycin (FeBLM), have been studied using EPR spectroscopy. It had been reported [Guajardo, R. J.; Hudson, S. E.; Brown, S. J.; Mascharak, P. K. *J. Am. Chem. Soc.* **1993**, *115*, 7971] that iron complexes of PMAH can be activated to a ferric hydroperoxide, a structural analog by activated BLM, by various routes, including reaction of $[Fe^{III}PMA]^{2+}$ with iodosylbenzene (PhIO), methanol, and base. We show that with or without PhIO, the latter reaction produces the methoxide complex of $Fe^{III}PMA$, and not the hydroperoxide, contrary to the previous report. Thus, O−O bond formation through generation of a ferric hydroperoxide does not occur. The same methoxide complex ($g = 2.28, 2.18, 1.93$) is generated by the addition of organic base and CH_3OH, or $LiOCH_3$, to $[Fe^{III}PMA]^{2+}$. The formation of this $[CH_3O-Fe^{III}PMA]^{+}$ complex is confirmed by EPR titration of $[Fe^{III}PMA]^{2+}$ with $^{-}OCH_3$ and by electrospray mass spectrometry. In contrast, the hydroperoxy complex of $Fe^{III}PMA$ ($g = 2.22, 2.17, 1.94$) can be generated by the reaction of $[Fe^{III}PMA]^{2+}$ with hydrogen peroxide or during aerobic oxidation of $[Fe^{II}PMA]^{+}$. The present results illustrate that activation of iron complexes of PMAH occur under conditions that produce activated BLM and further demonstrate that reaction of $[Fe^{III}PMA]^{2+}$ with PhIO and base does not provide a route to either a hypervalent iron species or a hydroperoxide intermediate. These results agree with the finding that PhIO cannot be used to generate activated bleomycin [Sam, J. W.; Tang, X.-J.; Magliozzo, R. S.; Peisach, J. *J. Am. Chem. Soc.* **1995**, *117*, 1012].

Introduction

Bleomycin (BLM) (Figure 1a) is an antitumor antibiotic used clinically in the treatment of various carcinomas and lymphomas.[1] The cytotoxic effect of bleomycin is believed to occur through oxidative degradation of DNA, a result of the drug's ability to bind iron, and subsequently bind and activate dioxygen.[2−5] The form of the drug kinetically competent in the in vitro cleavage of DNA, known as activated BLM, can be formed from ferrous BLM plus dioxygen and a single reducing equivalent, or from ferric BLM with H_2O_2.[6] It is now known that activated BLM is a ferric hydroperoxide complex,[6,7] $HOO-Fe^{III}$BLM, whose initial attack on DNA entails the abstraction of the 4′ hydrogen from the deoxyribose ring.[8,9]

Bleomycin possesses four main structural domains including a bithiazole group, a linker region, a metal binding (oxygen activation) domain, and a disaccharide moiety (Figure 1a). Recently, it has been suggested that the sequence specificity for DNA cleavage by FeBLM, with a preference for 5′-GpPy-3′ (Py = pyrimidine),[10,11] is governed by the metal binding domain.[12,13] Optical, resonance Raman, and EPR spectroscopic investigations of interactions of Fe^{III}BLM with DNA provide further evidence for the contribution of the metal binding site to sequence recognition.[14]

PMAH (Figure 1b) is a synthetic analogue of bleomycin whose structure mimics the metal chelating domain of the drug. According to a recent report by Guajardo et al.,[15] activation of Fe(II) and Fe(III) complexes of PMAH proceeds along routes similar to those for the formation of activated bleomycin.[6] Interestingly, the same sequence specificity for DNA cleavage by BLM and PMAH was also reported.[15] Furthermore, it was shown that $[Fe^{III}PMA]^{2+}$ with iodosylbenzene (PhIO) plus a single equivalent of LiOH in CH_3OH, generates a species whose EPR spectrum ($g = 2.28, 2.17, 1.93$)[15] is similar to that of activated BLM ($g = 2.26, 2.17, 1.94$).[6] It was therefore suggested that a low-spin Fe(III) hydroperoxy species, [HOO−

* To whom correspondence should be addressed.

(1) Umezawa, H. In *Bleomycin*; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; pp 24−36.
(2) Sausville, E. A.; Stein, R. W.; Peisach, J.; Horwitz, S. B. *Biochemistry* **1978**, *17*, 2746.
(3) Burger, R. M.; Peisach, J.; Blumberg, W. E.; Horwitz, S. B. *J. Biol. Chem.* **1979**, *254*, 10906.
(4) Burger, R. M.; Peisach, J.; Wittenberg, J. *J. Biol. Chem.* **1979**, *254*, 12299.
(5) Suzuki, H.; Nagai, K.; Yamaki, H.; Tanaka, N.; Umezawa, H. *J. Antibiot.* **1969**, *22*, 446.
(6) Burger, R. M.; Peisach, J.; Horwitz, S. *J. Biol. Chem.* **1981**, *256*, 11636.
(7) Sam, J. W.; Tang, X.-J.; Peisach, J. *J. Am. Chem. Soc.* **1994**, *116*, 5250.
(8) Wu, J. C.; Kozarich, J. W.; Stubbe, J. *J. Biol. Chem.* **1983**, *256*, 8606.
(9) Wu, J. C.; Kozarich, J. W.; Stubbe, J. *Biochemistry* **1985**, *24*, 7562.
(10) D'Andrea, A. D.; Hasseltine, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3608.
(11) Takeshita, M.; Grollman, A. P.; Ohtsubo, E.; Ohtsubo, H. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5983.
(12) Wu, W.; Vanderwall, D. E.; Lui, S. M.; Tang, X. J.; Turner, C. J.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1996**, *118*, 1268.
(13) Boger, D. L.; Ramsey, T. M.; Cai, H.; Hoehn, S. T.; Kozarich, J. W.; Stubbe, J. *J. Am. Chem. Soc.* **1998**, *120*, 53.
(14) Sam, J. W.; Takahashi, S.; Lippai, I.; Peisach, J.; Rousseau, D. L. *J. Biol. Chem.* **1998**, 273, 16090.
(15) Guajardo, R. J.; Hudson, S. E.; Brown, S. J.; Mascharak, P. K. *J. Am. Chem. Soc.* **1993**, *115*, 7971.

10.1021/ja980458p CCC: $18.00
Published on Web 11/25/1998

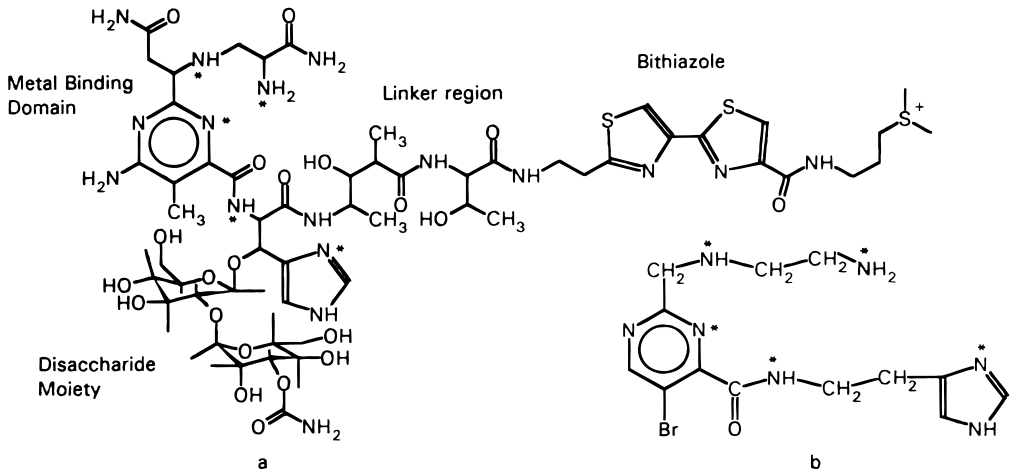

Figure 1. (a) Structure of bleomycin-A_2 with structural domains indicated. Asterisks denote presumed metal binding ligands. (b) Structure of PMAH. Asterisks denote ligands to iron.[15]

$Fe^{III}PMA]^+$, is formed from the reaction of $[Fe^{III}PMA]^{2+}$, PhIO, and ^-OH, as in eq 1:[15]

$$[Fe^{III}PMA]^{2+} + PhIO + {}^-OH \rightarrow [HOO-Fe^{III}PMA]^+ + PhI \quad (1)$$

The remarkable O−O bond formation represents the reverse of the reaction generally considered for other iron systems in which initial production of a ferric hydroperoxide is followed by heterolysis of the O−O bond, generating an iron oxene and hydroxide.[17]

The apparent behavior of $[Fe^{III}PMA]^{2+}$ lies in contrast to that of $Fe^{III}BLM$, since attempts to produce a species with the EPR spectrum of activated BLM using $Fe^{III}BLM$ and PhIO in the presence or absence of base, were unsuccessful.[18,19] Moreover, PhIO brings about the rapid degradation of $Fe^{III}BLM$.[19,20] In addition, electrospray mass spectrometry does not reveal the production of activated BLM from $Fe^{III}BLM$ with PhIO and base, although this technique was useful in detecting activated BLM formed from $Fe^{III}BLM$ with H_2O_2.[20]

To reconcile the discrepancy between the finding that $[Fe^{III}PMA]^{2+}$ can be activated with PhIO while $Fe^{III}BLM$ cannot, we reinvestigated the reaction products of $[Fe^{III}PMA]^{2+}$ with PhIO under the conditions originally described. Although a reaction mixture containing $[Fe^{III}PMA]^{2+}$, PhIO, CH_3OH, and base produces a species whose EPR signal is nearly identical to that of activated BLM, further investigation shows that this signal does not arise from a hydroperoxide, as in activated BLM, but rather from a low-spin ferric methoxy species. This rules out O−O bond formation from the oxygen of PhIO and ^-OH, as originally proposed.[15] The hydroperoxo analogue of activated bleomycin can instead be generated from $[Fe^{III}PMA]^{2+}$ and hydrogen peroxide (g = 2.22, 2.17, 1.94) or during aerobic oxidation of $[Fe^{II}PMA]^+$. These results do not contradict the claim that activated PMAH can be formed as an analogue of activated bleomycin, but serve to correctly identify the formation and EPR spectrum of this species.

(16) While Guajardo et al.[15] labeled this reaction the reversal of monooxygenase chemistry, implying the formation of an iron oxene preceding the formation of the ferric peroxide complex, Sauer-Masarwa et al. (Sauer-Masarwa, A.; Herron, N.; Fendrick, C. M.; Busch, D. H. *Inorg. Chem.* **1993**, *32*, 1086) argued that other mechanistic interpretations of this reaction are possible.

(17) Dawson. J. H. *Science* **1988**, *240*, 433 and references therein.

(18) Padbury, G.; Sligar, S. G.; Labeque, R.; Marnett, L. J. *Biochemistry* **1988**, *27*, 7846.

(19) Magliozzo, R. S.; Peisach, J. *Inorg. Chem.* **1989**, *28*, 608.

Experimental Section

Materials. PMAH (2-[[*N*-(aminoethyl)amino]methyl]-4-[*N*-[2-(4-imidazolyl)ethyl]carbamoyl]-5-bromopyrimidine) was prepared as previously reported[21] with the following modifications: (1) the reaction of 5-bromo-2-(hydroxymethyl)-4-pyrimidinecarboxylic acid with thionyl chloride was carried out in the presence of a small amount (0.1−0.2 equiv) of pyridine to catalyze the decomposition of the chlorosulfinate[22−24] intermediate (in the absence of pyridine, the reaction can take other courses[25]); (2) in the last step, 4 equiv of ethylenediamine, instead of 6, were added to the 2-(chloromethyl)-4-[2-(4-imidazolyl)ethyl]carbamoyl]-5-bromopyrimidine.

The ^1H and ^{13}C NMR data for PMAH are in agreement with those previously reported.[21] Electrospray mass spectrometry confirmed the appropriate mass of the product (m/z = 368.2 and 370.2 due to ^{79}Br and ^{81}Br isotopes with isotopic distribution of 50.54 and 49.46%).

Bleomycin sulfate (Blenoxane) was generously supplied by Bristol-Myers and used without further purification. A molecular weight of 1550 was assumed.[3]

$[Fe(DMSO)_6](ClO_4)_3$·DMSO and iodosylbenzene were synthesized according to published procedures.[26,27] $H_2{}^{17}O$ (86−89.9 atom % isotopic enrichment) was purchased from Isotec. All other reagents, including anhydrous solvents, were purchased from Aldrich.

Sample Preparation. $[Fe^{III}PMA]^{2+}$ was prepared by vigorous stirring of $[Fe(DMSO)_6](ClO_4)_3$·DMSO in CH_3CN with excess PMAH for 4 h under anaerobic conditions. The electronic absorption spectrum of $[Fe^{III}PMA]^{2+}$ was similar to that previously reported, and the yield was 93% based on the published extinction coefficient.[15] The identity of the product was confirmed by its EPR spectrum ($g_\perp$ = 2.28, $g_\parallel$ = 1.87) (Figure 2a).[15]

For iodosylbenzene experiments, EPR samples contained 6 mM $[Fe^{III}PMA]^{2+}$ and 9 mM LiOH in CH_3CN/CH_3OH mixtures[28] in an attempt to reproduce the experimental conditions of Guajardo et al.[15] PhIO in CH_3OH was added from a stock solution to a final concentration of 9 mM. The same reaction mixtures were used for ionspray mass spectrometry.

An EPR titration of $[Fe^{III}PMA]^{2+}$ (6 mM in CH_3CN) with $LiOCH_3$ (6 mM in CH_3OH) was carried out by continouosly varying the mole

(20) Sam, J. W.; Tang, X.-J.; Magliozzo, R. S.; Peisach, J. *J. Am. Chem. Soc.* **1995**, *117*, 1012.

(21) Brown, S. J.; Stephan, D. W.; Mascharak, P. K. *J. Am. Chem. Soc.* **1988**, *110*, 1996.

(22) Newman, M. S. *J. Am. Chem. Soc.* **1940**, *62*, 2295.

(23) Noller, C. R.; Bannerot, R. A. *J. Am. Chem. Soc.* **1934**, *56*, 1563.

(24) Gerrard, W. *J. Chem. Soc.* **1939**, 99.

(25) Bissinger, W. E.; Kung, F. E. *J. Am. Chem. Soc.* **1947**, *69*, 2158.

(26) Cotton, F. A.; Francis, R. *J. Am. Chem. Soc.* **1960**, *82*, 2986.

(27) Saltzman, H.; Sharefkin, J. G. *Org. Synth.* **1963**, *43*, 60.

(28) The solvent used by Guajardo et al.[15] was an unspecified mixture of CH_3CN and CH_3OH. We reacted $[Fe^{III}PMA]^{2+}$ with LiOH in the presence and absence of PhIO in CH_3CN/CH_3OH mixtures with v/v ratios of 1:1, 1.3:1, and 1:1.3.

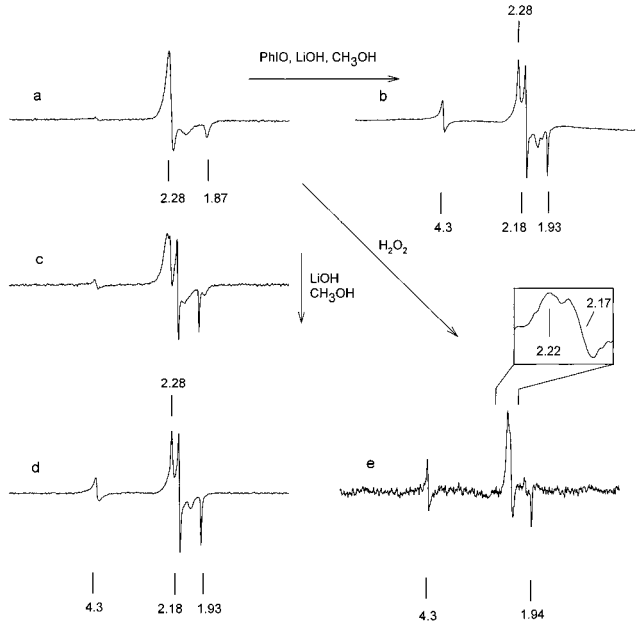

Figure 2. (a) X-band EPR spectra of 6 mM $[Fe^{III}PMA]^{2+}$ in CH_3CN. In b, 1.5 equiv of PhIO and of 1.5 equiv of LiOH in CH_3OH are added to a. In c, 0.75 equiv of LiOH in CH_3OH is added to a. In d, an additional 0.75 equiv of LiOH in CH_3OH is added to c. In e, excess H_2O_2 is added to a. Relevant g values are indicated. The resonance at $g = 4.3$ is attributed to high-spin ferric iron. Experimental conditions: microwave power 12.9 mW, modulation amplitude 4 G.

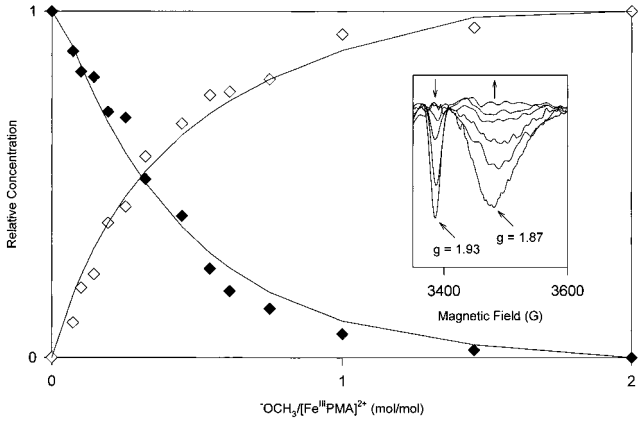

Figure 3. EPR titration of $[Fe^{III}PMA]^{2+}$ (6 mM in CH_3CN) with $LiOCH_3$ (6 mM in CH_3OH). ◆: $[Fe^{III}PMA]^{2+}$. ◇: $[CH_3O-Fe^{III}PMA]^{+}$. The data points in the Figure were obtained from the area under the $g = 1.87$ and $g = 1.93$ features of the EPR spectra for $[Fe^{III}PMA]^{2+}$ and $[CH_3O-Fe^{III}PMA]^{+}$, respectively (see inset).

ratio of the reagents in a constant volume monitoring both the production of $[CH_3O-Fe^{III}PMA]^{+}$ (followed at $g = 1.93$) and the disappearance of $[Fe^{III}PMA]^{2+}$ (followed at $g = 1.87$) (Figure 3). Precision EPR tubes were used for each sample.

Isotopic enrichment of $Li^{17}OH$ was achieved by dissolving 0.3 mg (0.0125 mmol) of anhydrous LiOH in 0.02 mL of $H_2{}^{17}O$ (minimum ^{17}O isotopic enrichment, 86.0−89.9%) followed by evaporation of solvent in a dry N_2 stream.

$[Fe^{II}PMA]^{+}$ was prepared by anaerobic addition of 0.009 g (0.045 mmol) of $FeCl_2 \cdot 4H_2O$, 0.482 g of (2.25 mmol) Proton-Sponge, and 5 mL of degassed anhydrous methanol to 0.023 g (0.063 mmol) of PMAH followed by vigorous stirring. The blue solution formed had no EPR signal, confirming the ferrous state of the complex.

$Fe^{III}BLM$ was generated by the addition of 1.1 equiv of BLM to a 1.5 mM solution of $FeCl_3 \cdot 6H_2O$ in anhydrous methanol in the presence of 25 equiv of Proton-Sponge. $Fe^{II}BLM$ was generated by the addition of 1.1 equiv of BLM to a 1.4 mM solution of $FeCl_2 \cdot 4H_2O$ in degassed anhydrous methanol in the presence of 20 equiv of Proton-Sponge. Samples used for EPR spectroscopy were diluted 1:1 with anhydrous, degassed toluene.

Anaerobic experiments were carried out under an ultrahigh purity grade N_2 atmosphere using degassed solvents.

Spectroscopic Measurements. EPR data were collected at 77 K on an X-band Varian E-112 spectrometer equipped with a Systron-Donner frequency counter using a personal computer and in-house data acquisition software (WINCWEPR). UV/vis spectra were acquired using a modified Cary 14DS spectrophotometer (Aviv Associates, Lakewood, NJ). NMR spectra were obtained with a Bruker-Spectrospin 300 spectrometer. All mass spectra were acquired on an API III triple-quadrupole mass spectrometer equipped with an ionspray interface (PE-Sciex, Thornhill, ON, Canada).

Results

We were intrigued by the claim of Guajardo et al.[15] that addition of a methanolic solution of PhIO and base to $[Fe^{III}PMA]^{2+}$ in CH_3CN forms $[HOO-Fe^{III}PMA]^{+}$, since PhIO is not capable of carrying out similar hydroperoxide formation with $Fe^{III}BLM$.[18−20] We therefore reexamined this reaction using conditions similar to those originally reported.[15] The starting material, $[Fe^{III}PMA]^{2+}$ in CH_3CN, exhibits an axial EPR spectrum with $g = 2.28$, $g = 1.87$ (Figure 2a). Anaerobic addition of PhIO, LiOH, and CH_3OH to $[Fe^{III}PMA]^{2+}$ brings about a color change from deep red to brown and yields a new ferric species with a low-spin rhombic EPR spectrum ($g = 2.28$, 2.18, 1.93) (Figure 2b), as originally reported.[15] However, addition of LiOH and CH_3OH in the *absence* of PhIO to an anaerobic solution of $[Fe^{III}PMA]^{2+}$ in CH_3CN produces the same spectrum (Figure 2d), clearly indicating that its formation is independent of PhIO. The generation of the same product in the latter case without any oxygen donor suggests that the rhombic EPR spectrum does not arise from a hydroperoxide as originally proposed.[15] The assignment to a hydroperoxide had been based on the observation of the same spectrum generated during the reaction of $[Fe^{II}PMA]^{+}$ with O_2 in methanol and in the above-mentioned PhIO reaction.[15]

Experiments were performed to identify the ferric complex giving rise to the EPR spectrum shown in Figure 2b,d. Despite the low concentration of ^{-}OH[29] the possibility that this low-spin species could be a hydroxo complex of $Fe^{III}PMA$ was ruled out since $Li^{17}OH$ (^{17}O 86−89.9%) in CH_3OH does not broaden the EPR spectrum at any feature (not shown), as would be expected for hyperfine interaction with the nuclear spin of bound ^{17}O ($I = {}^5/_2$). Such line broadening is seen, for example, in the EPR spectrum of $Mb^{17}OH$,[30] another low-spin ferric species with a rhombic EPR spectrum.

Another possibility is that this species is formed by deprotonation of $[Fe^{III}PMA]^{2+}$ by the added base. This is also ruled out because base added in the absence of CH_3OH does not alter the EPR spectrum of $[Fe^{III}PMA]^{2+}$ (not shown). Also, CH_3OH in the absence of base has no significant effect on the EPR spectrum of $[Fe^{III}PMA]^{2+}$, ruling out CH_3OH coordination (not shown).

It is suggested, instead, that here as well as under the conditions originally reported for the addition of PhIO, LiOH, and CH_3OH to $[Fe^{III}PMA]^{2+}$, $^{-}OCH_3$, generated[29] by the deprotonation of CH_3OH by LiOH, is bound to the metal ion and PhIO is not required for the reaction. The resulting $[CH_3O-Fe^{III}PMA]^{+}$ complex gives rise to a low-spin rhombic EPR

(29) The ^{-}OH concentration from LiOH is negligible because of the abundance of the dissociable protons from CH_3OH. This has been confirmed by ionspray mass spectrometry.

(30) Gupta, R. K.; Mildvan, A. S.; Schonbaum, G. R. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 1334.

Table 1. A Comparison of g Values for Hydroperoxy (HOO^-) and Methoxy (CH_3O^-) Complexes of $Fe^{III}BLM$ and $Fe^{III}PMA$

	HOO^-			CH_3O^-		
	g_z	g_y	g_x	g_z	g_y	g_x
$Fe^{III}BLM$	2.25	2.17	1.93	2.40	2.17	1.89
$Fe^{III}PMA$	2.22	2.17	1.94	2.28	2.18	1.93

spectrum (Figure 2b,d) with g values and line shape bearing resemblance to those of activated BLM (Table 1). Furthermore, the same rhombic EPR spectrum is also generated in the absence of PhIO or added ^-OH, when the organic base, *N*,*N*,*N*′,*N*′,-tetramethyl-1,8-naphthalenediamine (Proton-Sponge, Aldrich) in CH_3OH or when $LiOCH_3$ in CH_3OH is added to a solution of $[Fe^{III}PMA]^{2+}$ in CH_3CN (data not shown). In all instances, methoxide, either added directly or formed endogenously, ligates to $Fe^{III}PMA$.

To confirm the assignment of the low-spin rhombic EPR signal to $[CH_3O-Fe^{III}PMA]^+$, $[Fe^{III}PMA]^{2+}$ was titrated with $LiOCH_3$. Figure 3 shows the conversion of $[Fe^{III}PMA]^{2+}$ to the methoxy complex as a function of the $^-OCH_3/[Fe^{III}PMA]^{2+}$ ratio. The crossover point at 50% conversion indicates that only two species are present and that the loss of a single equivalent of $[Fe^{III}PMA]^{2+}$ yields 1 equiv of $[CH_3O-Fe^{III}PMA]^+$. Furthermore, no EPR signal intensity is lost, and thus, no reduction of $[Fe^{III}PMA]^{2+}$ occurs.

The in situ formation of $[CH_3O-Fe^{III}PMA]^+$ from $[Fe^{III}PMA]^{2+}$, LiOH, and CH_3OH is also confirmed by ionspray mass spectrometry (m/z = 453 and 455 corresponding to the 50.54 and 49.46% isotopic distribution of ^{79}Br and ^{81}Br in the PMAH molecule). The addition of PhIO does not affect this result nor is a hydroperoxide or iron oxene species detected.

The low-spin rhombic EPR signal (g = 2.28, 2.18, 1.93), originally assigned by Guajardo et al., to the hydroperoxide of $Fe^{III}PMA$, is also observed when $[Fe^{II}PMA]^+$ in methanol is exposed to air in the presence of base. To demonstrate that this signal does not arise from a hydroperoxo species, as in activated bleomycin, we investigated the effect of KI on its stability. KI, which immediately discharges activated BLM both in aqueous solution[31] and in methanol, as demonstrated here (Figure 4a,b),[32] has no effect on the EPR signal. Thus, the EPR active species initially formed from $[Fe^{II}PMA]^+$ and O_2 is not a hydroperoxide.

In the later stages of the reaction of $[Fe^{II}PMA]^+$ with O_2, another EPR signal (g = 2.21, 2.18, 1.93) appears, which predominates after 50 s and is rapidly discharged by KI. This species has nearly the same spectral features as that formed in the reaction of $[Fe^{III}PMA]^{2+}$ with hydrogen peroxide in acetonitrile (Figure 2e). We assign this EPR signal, formed either from ferrous or ferric PMA, to a hydroperoxide, a structural analogue of activated bleomycin.[7] Its formation from $[Fe^{II}PMA]^+$ however does not follow the same reaction pathway as that for activation of $Fe^{II}BLM$. In the latter case, oxygenated $Fe^{II}BLM$ oxidizes another $Fe^{II}BLM$ to form equimolar amounts of $Fe^{III}BLM$ and activated BLM. With $[Fe^{II}PMA]^+$ and O_2, the hydroperoxide appears after the formation of the methoxide complex of $Fe^{III}PMA$ (Figure 5).

It should be noted here that we have generated the methoxy and hydroperoxy adducts of both $Fe^{III}BLM$ and $Fe^{III}PMA$ under similar conditions (Table 1). The EPR spectra of the drug and model complexes bearing the same anionic ligand are not identical. The difference in anisotropy between the respective methoxy and the hydroperoxy forms of each is consistent with the relationship between the anionic ligand pK_a and the rhombicity of the low-spin EPR spectra, as noted by Sugiura.[33]

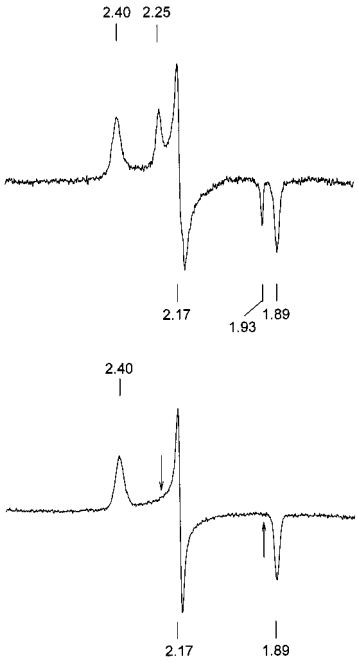

Figure 4. (a) X-band EPR spectrum of 1.4 mM $Fe^{II}BLM$ in methanolic solution containing 20 equiv of Proton-Sponge after exposed to air at 20 °C for 30 s. The EPR signal with g = 2.40, 2.17, 1.89 arises from $CH_3O-Fe^{III}BLM$ while that with g = 2.25, 2.17, 1.93 arises from $HOO-Fe^{III}BLM$ (activated bleomycin). (b) 200 equiv of KI in methanol was added to a followed by immediate refreezing. The two arrows indicate the g_x and g_z of activated bleomycin, shown above, which is discharged by KI, to form additional $CH_3O-Fe^{III}BLM$. Experimental conditions as in Figure 2. Spectrometer gain has been arbitrarily adjusted.

Methoxide has been previously reported as a ligand to ferric iron. It occurs in (meso-tetraphenylporphinato)iron(III) methoxide, which is formed from (meso-tetraphenylporphinato)iron(III) bromide,[34] with piperidine, in CH_3OH. The $^-OCH_3$ formed from the organic base and CH_3OH, replaces the axial Br^- ligand. In another case, facile formation of (methoxo)(octaethyl)(porphinato)iron(III) from the hydroxo species occurs in the presence of CH_3OH.[35] More recently, a methoxide ligand of ferric iron is also reported for a functional non-heme iron model of lipoxygenase.[36]

Discussion

Recently, synthetic analogues[15,37] of bleomycin were the subject of extensive and continued examination to define the fundamental functional role of individual structural domains of the drug associated with DNA binding and specificity of cleavage. Among these domains, the metal binding site of BLM is central to oxygen activation and sequence specificity. $[Fe^{III}PMA]^{2+}$ provides an analogue of the metal binding site of $Fe^{III}BLM$ where oxygen activation, to form a ferric hydroperoxide, occurs. A recent report by Guajardo et al.,[15] claimed that

(31) Burger, R. M.; Blanchard J. S.; Horwitz, S. B.; Peisach, J. *J. Biol. Chem.* **1985**, *260*, 15406.

(32) $CH_3O-Fe^{III}BLM$ was also formed and detected by EPR spectroscopy (g = 2.40, 2.17, 1.89) in the methanolic solution of $Fe^{III}BLM$ in the presence of base (data not shown).

(33) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5208.

(34) Lecomte, C.; Chadwick, D. L.; Coppens, P.; Stevens, E. D. *Inorg. Chem.* **1983**, *22*, 2982.

(35) Hatano, K.; Uno, T. *Bull. Chem. Soc. Jpn.* **1990**, *63*, 1825.

(36) Jonas, R. T.; Stack, T. D. P. *J. Am. Chem. Soc.* **1997**, *119*, 8566.

(37) Boger, D. L.; Teramoto, S.; Honda, T.; Zhou, J. *J. Am. Chem. Soc.* **1995**, *117*, 7338 and references therein.

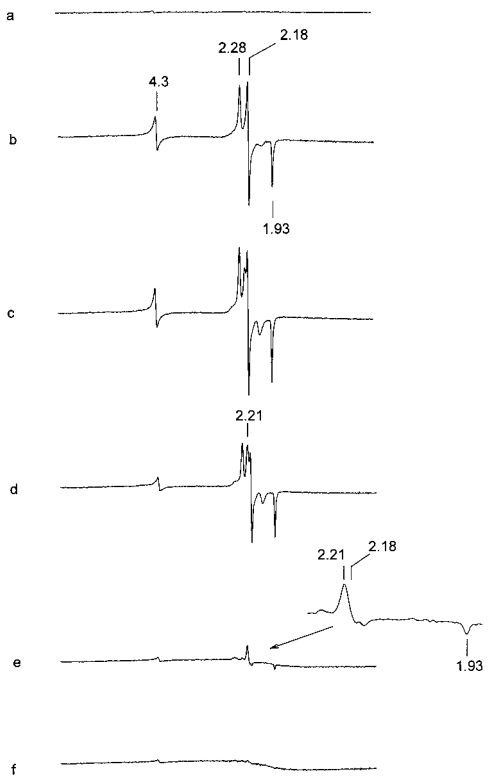

Figure 5. X-band EPR spectra showing the time course of the spectral changes observed when 9 mM $[Fe^{II}PMA]^+$ in methanolic solution containing 50 equiv of Proton-Sponge is exposed to air (a) before mixing with air; (b) sample was thawed, mixed with air and refrozen within 15 s; (c) sample b was thawed and refrozen within 15 s; (d) sample c was thawed and refrozen within 10 s; (e) sample d was thawed and refrozen within 10 s; (f) sample e was thawed and refrozen within 10 s. The EPR signal with $g = 2.28, 2.18, 1.93$ arises from $[CH_3O-Fe^{III}PMA]^+$ while that with $g = 2.21, 2.18, 1.93$ arises from $[HOO-Fe^{III}PMA]^+$. Note that the EPR spectrum of the methoxide complex b appears before that of the hydroperoxo complex e, shown for comparison in Figure 2e. After 50 s a red precipitate is formed and no EPR active species is seen (f).

$[Fe^{II}PMA]^+$, like Fe^{II}BLM, could be activated with O_2 to form a ferric hydroperoxide. For $[Fe^{III}PMA]^{2+}$, activation with PhIO and base was claimed to occur as well, although this reaction does not take place with Fe^{III}BLM.[18−20] The reaction mechanism suggested by Guajardo et al.[15] involved the addition of 1 equiv of $^-$OH to an equimolar mixture of $[Fe^{III}PMA]^{2+}$ and PhIO, which reportedly yielded the ferric hydroperoxide. For this reaction to occur, there are two or more potential intermediates that need be considered: an iodosylbenzene addition product of Fe^{III}PMA or a perferryl intermediate, an iron oxene species (Fe^V═O). We argue against the scheme of Guajardo et al. for hydroperoxide O−O bond formation (eq 1) by either route. No evidence was presented for the initial formation of an PhIO adduct or an oxene species since stoichiometric addition of PhIO to $[Fe^{III}PMA]^{2+}$ caused no change in the oxidation state of iron or coordination of oxygen, based on lack of significant changes in the EPR spectrum of $[Fe^{III}PMA]^{2+}$.[15] Furthermore, addition of a single equivalent of $^-$OH could not provide the driving force for the conversion of any postulated Fe^V═O intermediate to the ferric hydroperoxide oxidation level, based on the standard reduction potential of Compound I of horseradish peroxidase.[17,38] In any case, it is unlikely that an appropriate amount of free hydroxide remains in the methanolic solution of LiOH under the conditions used by Guajardo et al.[28,29] These arguments explain why the reported[15] EPR spectral changes occur after the addition of methanol and base and not upon addition of PhIO. Thus, no activation (hydroperoxo complex formation) occurs, the product instead being the methoxy complex of Fe^{III}PMA. A Fe(III) hydroperoxide species having a different EPR spectrum is in fact formed by the addition of H_2O_2 to $[Fe^{III}PMA]^{2+}$ and during aerobic oxidation of $[Fe^{II}PMA]^+$. This species, unlike the methoxide, is redox active with KI. Thus, these results assign new low-spin ferric forms of BLM and PMAH which had not been previously identified (Table 1) and refute the only spectroscopic demonstration of oxygen donor capabilities of iodosylbenzene to form an O−O bond.

Hypervalent iodine compounds[39−41] have been used in several systems to model oxygen activation by heme and non-heme iron. Iodosylbenzene, a well studied member of this class of reagents, is considered a single oxygen atom donor that directly generates hypervalent metal oxenes from their normal valent counterparts. Both Fe^{III}BLM and $[Fe^{III}PMA]^{2+}$ with PhIO were claimed to epoxidize olefinic substrates via formation of a hypervalent iron intermediate.[15,42] In contrast to olefin epoxidation, Fe^{III}BLM with PhIO does not carry out the DNA cleavage reaction characteristic of peroxide activated Fe^{III}BLM.[43,44] This observation argues against a common activated intermediate formed by PhIO or H_2O_2 with Fe^{III}BLM. The question is then raised what is the species responsible for the oxo transfers and epoxidations carried out in the presence of iodosylbenzene. Nam and Valentine[45] and Yang et al.[46] concluded that some metal-catalyzed iodosylbenzene reactions that had previously been assumed to proceed via high-valent metal oxo intermediates actually occur without the participation of redox active metals. More recently, it was found that, in the presence of light, epoxidation of *cis*-cyclooctene with PhIO occurs even in the absence of metals.[47] Our spectroscopic study rules out the formation of a ferric hydroperoxo species from $[Fe^{III}PMA]^{2+}$ with PhIO and supports the view that PhIO-mediated oxotransfer reactions may occur without the formation of a hypervalent metal intermediate. Furthermore, an apparent discrepancy between the behavior of Fe^{III}BLM and $[Fe^{III}PMA]^{2+}$ with PhIO has been clarified.

Acknowledgment. This investigation was supported by grants GM40168 and RR02583 from the United States Public Health Service (J.P.). We thank Wenyu Wang for the WINCWEPR data acquisition program, Dr. Mark Girvin for the use of the NMR facility, and Dr. Edward Nieves for providing the mass spectrometric measurements.

JA980458P

(38) Schulz, C. E.; Devaney, P. W.; Winkler, H.; Debrunner, P. G.; Doan, N.; Chiang, R.; Rutter, R.; Hager, L. P. *FEBS Lett.* **1979**, *103*, 102.
(39) Stang, P. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 274.
(40) Moriarty, R.; Prakash, O. *Acc. Chem. Res.* **1986**, *19*, 244.
(41) Banks, D. *Chem. Rev.* **1966**, *66*, 243.
(42) Murugesan, N.; Hecht, S. *J. Am. Chem. Soc.* **1985**, *107*, 493.
(43) Murugesan, N.; Ehrenfeld, G.; Hecht, S. *J. Biol. Chem.* **1982**, *257*, 8600.
(44) Ehrenfeld, G. M.; Rodrigez, L. O.; Hecht, S. M.; Chang, C.; Basus, V. J.; Oppenheimer, N. J. *Biochemistry* **1985**, *24*, 81.
(45) Nam, W.; Valentine, J. S.; *J. Am. Chem. Soc.* **1990**, *112*, 4977.
(46) Yang, Y.; Diederich, F.; Valentine, J. S. *J. Am. Chem. Soc.* **1990**, *112*, 7826.
(47) Samnani, P. B.; Bhattacharya, P. K. *J. Mol. Catal.* **1997**, *126*, L1.